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Award Number: DAMD17-03-1-0470

TITLE: Centrosome Amplification: A Potential Marker of Breast Cancer Agressiveness

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REPORT DATE: July 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-07-2006	Annual Summary	1 Jul 2003 – 30 Jun 2006

4. TITLE AND SUBTITLE

Centrosome Amplification: A Potential Marker of Breast Cancer Agressiveness

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0470

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Antonino B. D'Assoro, M.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mayo Clinic
Rochester, MN 55902

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

The aim of our research is focused in elucidating the mechanisms by which the normal regulatory pathways coordinating centrosome duplication with cell cycle events may become uncoupled promoting breast cancer development, progression, chemoresistance and consequent poor outcome. The preliminary results reported in this grant suggest that the development and progression of breast cancer is a complex process involving the role of estrogens, growth factor signaling pathways and abrogation of the p53 protein leading to an inactivation of cell cycle checkpoints. We have demonstrated that although MCF-7 cells stable transfected with a dominant-negative p53 construct, maintain estrogen-dependent properties, the timing of centrosome duplication and cyclin/cdk complexes is deregulated following mitogen stimulation. Interestingly, over-expression of cyclin A plays a critical role in the development of centrosome amplification following hormone stimulation. We also have shown that genotoxic stress leads to centrosome amplification in MCF-7 breast cancer cells with mutant p53, but not in MCF-7 cells over-expressing oncoproteins in the EGF mitogen signaling pathway with wild-type p53 background. Our findings demonstrate that over-expression of EGF mitogen signaling proteins is not sufficient to induce centrosome amplification following genotoxic stress, conferring to p53 a key role in the control of centrosome homeostasis and genomic stability. They also suggest that chemotherapy agents inducing DNA damage may lead to the selection of resistant clones through centrosome amplification only in cells with mutant p53 regardless over-expression of the EGF signaling pathway.

15. SUBJECT TERMS
Breast Cancer

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	37	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Breast cancer progression is characterized by the stepwise accumulation of heterogeneous genetic defects originating from the development of genomic instability [1]. One of the most common mechanisms for the origin of genomic instability in breast cancer is through flux in karyotype or chromosomal instability (CIN), which can give rise to aneuploidy [2]. CIN describes the rate of changes in chromosome number, while aneuploidy is characterized as the state of an altered chromosome number [3, 4]. Strong evidence supports the hypothesis that the level of chromosomal instability increases during tumor progression leading to the generation of cancer cell clones with more aggressive properties [5]. For this reason, the development of chemoresistance, linked to cancer progression, remains the principal obstacle for the complete eradication of cancer cells, and new markers with prognostic/predictive value are urgently needed to tailor treatment of patients with advanced breast cancer, improving their overall survival.

The development of breast cancer is driven by aberrant cross talk between estrogen and growth factor signaling pathways [6, 7]. Early breast cancer is usually characterized by an estrogen receptor (ER) positive phenotype. ER positive breast carcinomas generally have a better prognosis than ER negative tumors because they show hormone dependence to growth and low grade of chromosomal instability [8]. For this reason, the ER status represents an important predictive factor in tailoring ER + patients for treatment with anti-estrogens. However during tumor progression, breast carcinomas show an inclination to lose the estrogen receptor and acquire a hormone independent and more aggressive behavior [9]. Taken together, these findings suggest that the phenotype of advanced breast carcinomas differs from the primary tumor and furthermore that a selection of cancer cell clones with high metastatic potential occurs during tumor progression.

Despite the recent progress in early diagnosis and treatment, advanced breast cancer still remains an incurable disease with a median survival of 11 months [10]. Adjuvant treatment with antiestrogens and cytotoxic drugs reduces mortality in early breast cancer; however, this reduction is only 8-37%, and most patients are not cured by their adjuvant therapy [11]. Most cytotoxic drugs used in the management of breast cancer induce DNA damage and trigger cell cycle arrest and apoptotic cell death [12]. Usually, combination of different agents (polychemotherapy) such as cyclophosphamide, methotrexate and 5-fluorouracil (CMF) is preferred to a single agent in order to allow the use of lower doses, thereby decreasing toxicity with an improvement in therapeutic efficacy [13]. However, the principal failure of current chemotherapy in the treatment of breast cancer resides in the development of resistance that characterizes tumor progression [14]. The acquisition of resistance to cytotoxic agents in breast cancer is usually associated with the overexpression of multidrug resistance protein 1 (MDR1), c-ErbbB2 receptor amplification and/or inhibition of apoptotic pathways during tumor progression [14-16]. Acquisition of chemoresistance may also be linked to the high level of chromosomal instability as a source of clonal heterogeneity commonly observed in advanced breast carcinomas. Exposure of a breast tumor to cytotoxic agents may result in variable shrinkage, no change, or continuous tumor growth, depending on the number of sensitive and resistant cells within a heterogeneous population [17]. However, little is known about the molecular mechanisms leading to chromosomal instability and its role in the development of chemoresistance and tumor progression.

Emerging evidence suggests that the centrosome plays an essential role in the control of genomic stability through the establishment of the bipolar mitotic spindle and balanced segregation of chromosomes during cell division [4, 18, 19]. This mechanism ensures the propagation of a normal diploid chromosome number to daughter cells during mitosis. In order to maintain a normal diploid phenotype, the centrosome must be duplicated once, and only once, during each cell cycle to give rise to two centrosomes that function as the spindle poles of the dividing cell [20]. Coordination between centrosome duplication and DNA replication during the G1/S transition of the cell cycle is extremely important to maintain the integrity of the genome [21-23]. This process is accurately monitored through the phosphorylation status of the tumor suppressor retinoblastoma (Rb) and consequent activation of E2F transcription factor [23]. Furthermore, it has been

demonstrated that association of cyclin E and cyclin A with cyclin dependent kinase 2 (cdk2) stimulate centrosome duplication, while activation of the p53 pathway has an inhibitory effect on the centrosome duplication cycle [24-26]. Interestingly, loss of p53 function and/or hyperactivity of cyclin/cdk complexes lead to centrosome amplification, a pathological condition characterized by the presence of more than two centrosomes within a single cell [27]. Furthermore, overexpression of Polo-like kinase 1(Plk1) and Aurora-A centrosome kinases that control centriole duplication, centrosome maturation and cytokinesis has also been associated with the development of centrosome amplification and chromosomal instability in cancer [28, 29]. Taken together, these findings demonstrate that loss of centrosome homeostasis depends on the unbalance between oncogenes and tumor suppressors controlling cell cycle checkpoints and genomic integrity.

The link between centrosome amplification and the pathogenesis of cancer is mediated through the formation of multipolar mitotic spindles and consequent unequal chromosome segregation [30]. This mechanism is associated with the development of chromosomal instability and aneuploidy in cancer cells [31, 32]. These studies are supported by the observation that centrosome amplification occurs exclusively in aneuploid tumors and tumor derived cell lines in contrast to diploid tumors that show normal centrosomes [4, 33]. In addition, recent studies on centrosome amplification have shown that the degree of aneuploidy parallels the degree of centrosome abnormalities in breast cancer cell lines as well in human breast tumor tissues, and suggest a direct role of centrosome amplification in driving chromosomal instability during tumor progression [4, 34].

Recently, we investigated the relationship between induction of genotoxic stress by anticancer drugs, cell cycle checkpoint integrity and centrosome amplification in human breast cancer cell lines [22]. These findings established that introduction of DNA damage by genotoxic anticancer drugs leads to centrosome amplification in breast cancer cells with an abrogated G1/S cell cycle checkpoint. Bennett and coworkers [35] also demonstrated that commonly used chemotherapeutic agents targeting the DNA replication process lead to centrosome amplification in adult skin fibroblasts derived from mice lacking p53 (p53 MSFs). In addition, when these cells were released from cell cycle arrest by removal of drugs, the phenotype of these cells was characterized by extensive chromosomal instability. Although these studies were only carried out in cultured cells, they suggest that gain of centrosome amplification and consequent chromosomal instability following genotoxic stress may represent a key process in selecting highly aggressive clones responsible of recurrence, chemoresistance, metastatic spread to distant organs and consequent poor outcome.

The aim of our research is focused in elucidating the mechanisms by which the normal regulatory pathways coordinating centrosome duplication with cell cycle events may become uncoupled promoting breast cancer development, progression, chemoresistance and consequent poor outcome. Since negative (tumor suppressors) and positive (oncogenes) regulators of cell cycle progression can also regulate centrosome duplication, we propose that in breast cancer, alterations in growth factor signaling pathways, and/or inactivation of the p53 pathway act to inactivate G1/S and/or G2/M cell cycle checkpoints thereby promoting the development of an amplified centrosome phenotype. Centrosome amplification could represent an early event in breast cancer development, but the degree of amplification may also increase during tumor progression accelerating the rate of chromosomal instability and aggressiveness. We believe that this studies will have a strong impact in basic and clinical oncology for two reasons: first, they will help to define the molecular mechanisms leading to centrosome amplification in breast cancer; second, they will clarify if centrosome amplification represents a driving force in selecting hormone-independent and chemoresistant clones in breast cancer and its potential role as a new suitable prognostic marker of tumor aggressiveness and therapeutic target.

KEY RESEARCH ACCOMPLISHMENTS

1. **Abrogation of wild-type p53 function is associated with deregulation of cyclinA/CDK2 activity, a shortened G1/S phase progression and centrosome amplification following starvation and mitogen stimulation in MCF-7 breast cancer cells.**

2. **Genotoxic stress induces centrosome amplification in human breast cancer cells lacking the G1/S cell cycle checkpoint.**

3. **Hyperactivity of the EGF signaling pathway leads to over-expression of G1/S cyclins but is not sufficient to induce centrosome amplification following DNA damage in the presence of wild-type p53 in the MCF-7 cells.**

4. **Conventional chemotherapeutic agents lead to over-expression of Aurora-A centrosome kinase associated with centrosome amplification in breast cancer cells lacking p53 function.**

Specific aim #1: Determine the role of 17-B Estradiol and EGF growth factor receptor activation in the regulation of centrosome duplication. We will test the hypotesis that estrogen receptor and growth factor signaling are mechanistically coupled to centrosome duplication during G1/S progression of the cell cycle.

Breast cancer represents a hormone-dependent disease, estrogens play an essential role in the onset of breast cancer, however, the hormone-dependent phenotype is commonly lost during tumor progression. The EGF growth factor pathway plays a key role not only in the development of breast cancer but also in the progression of the disease since advanced breast cancers often over-express the EGF receptor and shows an hormone-independent and chemoresistant phenotype. The goal of this first aim is to determine the role that 17-Beta

estradiol and EGF growth factor pathways play in the regulation of centrosome duplication. We propose to develop and validate an in vitro model system for centrosome duplication that can be used to dissect the signaling events that are required for the development of centrosome amplification in breast cancer. Our preliminary results demonstrate that in the MCF-7 breast cancer cell line, 17-B estradiol and EGF stimulate centrosome duplication and this process is well coordinated with the expression of key cell cycle regulators (cyclin D, E, A, B,) and with the progressive phosphorylation status of the retinoblastoma tumor suppressor. Furthermore, in order to understand if abrogation of the p53 pathway may deregulate the centrosome duplication cycle in the MCF-7 cells stimulated with mitogens, we generated an MCF-7 cell line stable expressing a p53 dominant-negative construct (MCF-7Dnp53) to mask the function of the wild-type p53. Interestingly, the MCF-7Dnp53 still retained an estrogen dependent phenotype, since treatment with the anti-estrogen Tamoxifen inhibited the percentage of cells in the S phase of the cell cycle. However, when the MCF-7Dnp53 cells were stimulated with estradiol and EGF, they exhibited a deregulation in the timing of cyclin E and A abundance compared with the parental cell line that was associated with high retinoblastoma phosphorylation. Interestingly, the timing of cyclin D expression following hormone stimulation was maintained in the MCF-7Dnp53 cells. In order to link the acceleration of cell cycle progression in the MCF-7Dnp53 cells with centrosome amplification, we analyzed the centrosome phenotype in the MCF-7Dnp53 and the parental cell line in starvation and following mitogen stimulation. Centrosomes were stained with centrin and pericentrin antibodies and the percentage of cells with more than four centrioles were determined by immunofluorescence. Only the MCF-7Dnp53 cells developed amplified centrosomes, suggesting that development of centrosome abnormalities in breast cancer cells stimulated with mitogens requires inactivation of the p53 pathway with important consequences on the G1/S timing progression.
Interestingly, centrosome amplification in the MCF-7Dnp53 cells lead to the development of aberrant mitoses and consequent chromosomal instability following hormone stimulation. These findings suggest that estrogen receptor and EGF signaling pathways are mechanistically coupled to centrosome duplication through the orderly expression of G1/S cyclins only in the presence of wild-type p53. In conclusion, we propose a model of multi-step neoplastic transformation where estrogens and growth factors initiate the development of breast cancer and the consequent abrogation of the p53 pathway during tumor progression promotes the development of centrosome amplification leading to aberrant mitoses, chromosome instability and consequent poor out-come. **A manuscript elucidating these findings has been submitted to Cancer Research.**

Specific aim #2: Determine the relationship between cell cycle checkpoints and centrosome amplification. We will experimentally disrupt cell cycle progression in breast tumor-derived cell lines to test the hypothesis that G1/S and G2/M cell cycle checkpoints are mechanistically coupled to centrosome duplication, and that this linkage becomes uncoupled during mammary tumorigenesis.

The goal of this second aim is understand the molecular mechanisms linking inactivation of the G1/S and G2/M cell cycle checkpoints to centrosome amplification in breast cancer. We will address this question under three different experimental conditions: 1) we will arrest cell cycle progression in G1/S or G2/M to determine the role of these checkpoints in controlling centrosome duplication. 2) We will use specific inhibitors of cell cycle regulatory proteins to identify the key targets involved in this control mechanism. And finally, 3) We will generate clones of breast cancer cell lines with altered expression or activity of cell cycle regulators that will help us in defining their role in the control of centrosome homeostasis. The first part of our research has been devoted in understanding the role of the G1/S checkpoint in the control of centrosome homeostasis. Our results demonstrate that induction of genotoxic stress induces centrosome amplification in breast cancer cell lines with an abrogated G1/S cell cycle checkpoint (22). We used breast cancer cell lines with different p53 backgrounds to investigate the relationship between DNA damage, G1/S cell cycle checkpoint integrity, and the development of centrosome amplification. Introduction of DNA damage in the MCF-7 cell line by treatment with hydroxyurea (HU) or daunorubicin (DR) resulted in the arrest of both G1/S cell cycle progression and centriole duplication. In these cells, which carry functional p53, HU treatment also led to nuclear accumulation of p53

and p21WAF1, retinoblastoma hypophosphorylation, and downregulation of cyclin A. MCF-7 cells carrying a recombinant dominant-negative p53 mutant (vMCF-7DNp53) exhibited a shortened G1 phase of the cell cycle and retained a normal centrosome phenotype. However, these cells developed amplified centrosomes following HU treatment. The MDA-MB 231 cell line, which carries mutant p53 at both alleles, showed amplified centrosomes at the outset, and developed a hyperamplified centrosome phenotype following HU treatment. In cells carrying defective p53, the development of centrosome amplification also occurred following treatment with another DNA damaging agent, DR. Taken together, these findings demonstrate that loss of p53 function alone is not sufficient to drive centrosome amplification, but plays a critical role in this process following DNA damage through abrogation of the G1/S cell cycle checkpoint. Interestingly, we were able to reactivate the G1/S checkpoint and revert the centrosome phenotype in the MDA-MB 231 cell line following genotoxic stress by using a potent cdk inhibitor, Roscovitine. The novelty of these results consist in showing for the first time a clear relationship between DNA damage, abrogation of the G1/S checkpoint and the development of centrosome amplification arising important clinical implications regarding the management of breast cancer patients: first, they suggest that breast cancers with compromised p53 function may develop centrosome amplification and consequent chromosomal instability following treatment with genotoxic anticancer drugs. Second, to overcome the complications associated with centrosome amplification for this subset of breast cancer patients, treatment with cdk inhibitors in combination with anticancer genotoxic drugs may provide a new attractive therapeutic approach. In order to establish if deregulation of the EGF signaling pathway leads to centrosome amplification following genotoxic stress, Dr. McCubrey at the East Caroline University provided to our laboratory MCF-7 cells over-expressing the oncoproteins RAF-1 and V-Erbb2 but still retaining wild-type p53. To determine if genotoxic stress lead to centrosome amplification, MCF-7 RAF-1 and MCF-7 v-Erbb2 were incubated with HU and DR for 48 hours. The percentage of cells in each phase of the cell cycle before and after treatment was determined by FACS, the expression of key cell cycle checkpoint regulators was determined by immunoblotting and the centrosome phenotype was characterized by immunofluorescence using centrin and pericentrin antibodies. Our preliminary results clearly demonstrated that in the presence of wild-type p53, the MCF-7 RAF-1 and MCF-7 v-Erbb2 don't show centrosome amplification. The resulting centrosome phenotype was associated with the activation of the G1/S cell cycle checkpoint characterized, at molecular level, by over-expression of p53, $p21^{WAF1}$ and decreased phosphorylation of retinoblastoma. Interestingly, introduction of a Dnp53 construct in the MCF-7 RAF-1 cells induced centrosome amplification following genotoxic stress. These results suggest that over-expression of the EGF signaling pathway is not sufficient to induce centrosome amplification following DNA damage in the presence of wild-type p53. To validate our model in vivo, we employed nude mice to develop MCF-7RAF-1 and MCF-7RAF-1/Dnp53 xenografts. Since clinical studies have demonstrated that over-expression of the EGF receptor is associated with chemoresistance, we propose a model of chemoresistance as a multi-step process where the association between hyperactivity of EGF signaling pathway and loss of p53 induces centrosome amplification following treatment with anticancer drugs, conferring not only chemoresistance but also chromosomal instability with consequent poor out-come.

A manuscript elucidating these findings is currently in preparation.

Since our studies strongly suggest that chemotherapeutic agents may induce centrosome amplification in cancer cells lacking the cell cycle checkpoints, we treated the human breast cancer cell lines (MCF-7, MCF-7Dnp53, MCF-7 RAF-1, MCF-7 v-Erbb2 and MDA-MB 231) with chemotherapeutic agents employed in the treatment of breast cancer (5-Fluorouracil, Methotrexate, Doxorubicin, Cisplatin, Etoposide and Paclitaxel). Our studies demonstrated that following genotoxic stress, centrosome amplification was also linked to Aurora-A over-expression in the breast cancer cell lines lacking functional p53. Knock-down of Aurora-A by an expression vector over-expressing siRNA inhibited centrosome amplification following DNA damage. These studies suggest that Aurora-A may represent a novel molecular therapeutic target to inhibit centrosome amplification and chromosomal instability following treatment with chemotherapeutic agents in aggressive breast carcinomas with compromised cell cycle checkpoints. **A manuscript elucidating these findings is currently in preparation.**

REPORTABLE OUTCOMES

AACR Annual Meeting, April 16-20 Anaheim, CA

Genotoxic stress leads to centrosome amplification in breast cancer cell lines that have an inactive G1/S cell cycle checkpoint

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Centrosome amplification plays a key role in the origin of chromosomal instability through the establishment of multipolar mitoses and unequal chromosome segregation. However, the molecular mechanisms responsible for the development of centrosome amplification are not well understood. In this study, we used breast cancer cell lines with different phenotypes to investigate the relationship between genotoxic stress, activation of the G1/S checkpoint and centrosome amplification. Centrosome amplification was not seen in the MCF-7 cell line with wild-type p53 following genotoxic stress. These cells showed activation of the G1/S checkpoint indicated by upregulation of p21/waf1, retinoblastoma hyperphosphorylation and downregulation of cyclin A. In contrast, MCF-7 cells with abrogated p53 function and the MDA-MB 231 cell line amplified their centrosome upon introduction of DNA damage. In these cells centrosome amplification was linked to inactivation of the G1/S checkpoint. Furthermore, addition of roscovitine, a potent cdk inhibitor, to genotoxic agents inhibited centrosome amplification by rescuing the G1/S checkpoint in the MDA-MB 231 cell line. Interestingly, to test if centrosome amplification was strictly dependent on the loss of p53 function, we utilized an MCF-7 cell line expressing a constitutively active RAF-1 protein but still retained a wild-type p53 phenotype. Following genotoxic stress these cells developed a partially amplified centrosome phenotype indicating that loss of p53 plays a major role in the development of centrosome amplification. Taken together our results demonstrate that anticancer drugs targeting the DNA replication process induce centrosome amplification only in subtypes of breast cancer cells lacking the G1/S checkpoint. In addition, these studies provide a therapeutic rationale to inhibit centrosome amplification and chromosomal instability by using cdk inhibitors in association with conventional chemotherapeutic agents.

Manuscript sent for Revision to Cancer Research

Regulation of the timing of centrosome duplication following 17-β estradiol, EGF and IGF-I stimulation requires functional p53 in MCF-7 breast cancer cells

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Abstract

Maintenance of normal breast epithelial cells depends on the balance between mitogen and tumor suppressor signaling pathways. One of the mechanisms responsible for the origin of phenotypic heterogeneity commonly observed in breast tumors is the development of centrosome amplification leading to mitotic spindle abnormalities and consequent chromosomal instability. However, how mitogen and tumor suppressor pathways are mechanistically coupled to the centrosome cycle in breast cancer cells has not been established. In this study we show that in MCF-7 cells, hormone withdrawal led to arrest of DNA replication and centriole duplication, inhibition of G1/S cyclins activity and retinoblastoma (Rb) hypo-phosphorylation. Stimulation of arrested cells with 17-β estradiol, EGF and IGF-I, led to their orderly progression through the cell cycle characterized by the sequential expression of G1/S cyclins, progressive Rb phosphorylation and the timing of centriole duplication corresponding to G1/S transition. In contrast, MCF-7 cells with abrogated p53 function (vMCF-7^{Dnp53}) showed uncoupling of DNA replication and centriole duplication following hormone withdrawal. After mitogen stimulation, these cells showed shortened G_1/S cell cycle progression compared to the parental cell line. Importantly, while normal timing of cyclinD1 expression was maintained in these cells, their accelerated cell cycle progression was linked to premature expression of cyclin E and A, Rb hyper-phosphorylation and centrosome amplification. Furthermore, these cells also showed a high frequency of pseudobipolar and multipolar mitotic spindles. In conclusion, this study demonstrates that mitogen-signaling pathways coordinate centrosome duplication and cell cycle progression through the integrity of p53 function in hormone dependent breast cancer cells.

Introduction

The progression of human breast cancer from an estrogen dependent to an estrogen independent phenotype represents a major clinical problem that limits the long term usefulness of endocrine therapeutic strategies (1, 2). Among estrogens, 17-β estradiol is the major promoter of cell proliferation in both normal and neoplastic breast epithelium through its binding to high-affinity estrogen receptor (ERα) (3, 4). ERα functions as an estrogen activated transcription factor and mediates the stimulation of estrogen target genes involved in the regulation of cell proliferation and inhibition of apoptosis of the breast epithelium (5-7). Excessive stimulation of the ERα pathway due to increased hormonal secretion, prolonged exposure to estrogens or increased levels of the receptor may lead to deregulation of cell proliferation and thus increase the risk to develop breast cancer (8, 9). In addition to estrogens, growth factors, such as EGF and IGF-I act in an autocrine and/or paracrine fashion to induce the proliferation of breast epithelial cells (10). It has been demonstrated that aberrant cross-talk between estrogens and growth factors signaling pathways play an important role in the development and progression of breast cancer (11-13).

Chromosomal instability represents a hallmark of breast cancer and is responsible for the evolution of cancer cells with more aggressive behavior (14, 15). Recent studies show that development of centrosome amplification drives chromosomal instability and the consequent phenotypic heterogeneity of breast tumors, highlighting the centrosome as a key organelle in the control of chromosomal stability (15-17). The correct timing of centrosome duplication during cell cycle progression plays a critical role in the maintenance of a diploid karyotype (18, 19). In order to ensure the formation of a bipolar mitotic spindle leading to equal chromosome segregation during cytokinesis, centrosome duplication must be strictly coordinated with DNA replication during the G_1/S progression of the cell cycle generating no more than two centrosomes at the end of the G_2 phase (20-22). Breast tumors as well as breast cancer cell lines often show multiple centrosomes (centrosome amplification) linked to the formation of aberrant mitotic figures (23, 24). Aberrant mitoses resulting in unbalanced chromosome segregation, promote the generation of aneuploid cancer cells (25, 26). Inhibition of centrosome reduplication depends on the balance between oncogene and tumor suppressor activities (27). The tumor suppressors p53 and retinoblastoma inhibit centriole over-duplication (28, 29), while

over-expression of cyclin/cdk2 complex and the centrosome associated kinases Aurora-A and PLK1 promote the development of amplified centrosomes (30-34). Recent studies also demonstrate that anticancer genotoxic agents induce centrosome amplification and chromosomal instability due to the uncoupling of DNA replication and centriole duplication in cancer cells lacking p53 function (35, 36). These findings suggest that p53 controls centrosome homeostasis through activation of the G_1/S cell cycle checkpoint that monitors genomic integrity. However, whether p53 also plays a role in monitoring centrosome duplication following mitogen stimulation has not been established.

In this study, we investigated the role of 17-β estradiol, EGF and IGF-I in the regulation of the timing of centrosome duplication in the MCF-7 breast cancer cells with endogenous wild-type p53, or in these cells engineered to over-express a mutant p53 construct (vMCF-7^{DNp53}). Our findings demonstrate that in hormone stimulated MCF-7 cells with wild-type p53, centriole duplication is well coordinated with DNA replication. On the contrary, hormone stimulated MCF-7 cells with abrogated p53 function showed a deregulated G_1/S cell cycle progression characterized by premature expression of cyclin E and A and Rb hyper-phosphorylation. Interestingly, these cells also developed centrosome amplification leading to an increased number of pseudobipolar and multipolar mitotic spindles. In conclusion, the results presented here highlight an important role for p53 in maintaining centrosome homeostasis and mitotic fidelity in hormone stimulated breast cancer cells.

Materials and Methods

Human Breast Cancer Cell Lines: The human breast cancer cell line MCF-7 was obtained from ATCC (Manassas, VA, USA). The MCF-7 cells expressing a GFP-centrin2 chimera (MCF-$7^{GFP\text{-}cetn2}$) and variant MCF-7 cells expressing a dominant-negative p53 construct (vMCF-7^{DNp53}) were generated in our laboratory as described previously (35, 37). All the cell lines were maintained in EMEM medium containing 5mM glutamine, 1% penicillin/streptomycin, 20 microgram insulin/ml and 10% FBS at 37 C in 5% CO2 atmosphere. The vMCF-7^{DNp53} and MCF-$7^{GFP\text{-}cetn2}$ cells were also grown in the presence of 500 μg/ml G148.

Hormonal Treatment: To investigate the effects of 17-β estradiol, EGF and IGF-I on cell cycle progression, expression of G_1/S cyclins and centriole duplication, MCF-7 $^{GFP\text{-}cetn2}$ and vMCF-7^{DNp53} cells were allowed to proliferate for 2 days in complete medium, after which the medium was removed, and cells were washed twice in 1xPBS. Complete medium was replaced with a defined starvation medium: serum-free phenol red-free EMEM medium supplemented with 5% charcoal-stripped FBS serum and 2mM L-Glutamine. Starvation medium was changed once daily for 2 days. After 48h of starvation, cells were washed in 1x PBS and incubated with 10nM 17-β estradiol, 10ng/ML EGF and 10ng/ML IGF-I for 4, 8, 12, 24, 36 and 48 hours.

Cell cycle profile: For fluorescence-activated cell sorting (FACS) analysis, MCF-7 $^{GFP\text{-}cetn2}$ and vMCF-7^{DNp53} cells were washed with cold PBS, fixed in 95% ethanol, stained with propidium iodide overnight and analyzed by flow cytometry using Facscan by Becton Dickinson (Franklin Lakes, NJ, USA). The resulting cell cycle profiles, based on 20,000 events, were analyzed using the ModFit program using Verity Software House (Topsham, ME, USA). Experiments were performed in duplicate with similar results.

Centriole Duplication Assay: In order to determine the timing of centriole duplication following hormone stimulation, MCF-7$^{GFP\text{-}cetn2}$ cells were grown at a density of $3x10^5$ on glass cover slips. After two days the complete medium was replaced with starvation medium changed once daily for 2 days. After 48h of starvation, cells were washed in 1x PBS and incubated with 10nM 17-β estradiol, 10ng/ML EGF and10ng/ML IGF-I. For centrioles counting, MCF-7$^{GFP\text{-}cetn2}$ cells were fixed in 4% formaldehyde for one hour, washed in 1 X PBS, DNA was stained with Hoescht dye at 1µg/ml, and mounted using ProLong antifade (Molecular Probes). Images were captured using a Hamamatsu CCD camera at 30-second intervals for 10-20 min. and processed using Metamorph Imaging System software (Universal Imaging Corp., Westchester, PA). The values reported represent the average of 100 cells in each of two independent experiments.

Indirect Immunofluorescence: For indirect Immunofluorescence analysis, cells were grown on cover slips at a density of $3x10^5$, fixed in absolute methanol at –20 C for 10 min, blocked in 5% normal goat serum, 1% glycerol, 0.1% BSA, 0.1% fish skin gelatin, 0.04% sodium azide and incubated with primary antibodies. For the characterization of centrosome phenotype and mitotic spindle morphology following hormonal treatment, we used antibodies against the proteins centrin (centrin 2.4 produced in our laboratory) pericentrin

(Covance) and Aurora-A (Santa Cruz). Cover slips were washed three times in PBS followed by incubation with secondary antibodies conjugated with Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR, USA), washed three times in PBS and finally incubated in Hoescht dye at 1µg/ml to stain DNA. Immunofluorescence images were digitally recorded using a Zeiss fluorescence microscope equipped with computer-controlled focus, CCD digital camera, apotome system and Axiovision software. Fields were recorded at multiple focal planes and analyzed and printed as maximum projections to assure that all centrioles and centrosomal structures were imaged. The values reported represent the average of 100 cells in each of two independent experiments.

Immunoblotting: For immunoblot analysis, 20 µg protein of whole-cell lysate were run in 12% SDS-PAGE, transferred to PVDF membrane, fixed in 0.25% glutaraldehyde, blocked in 5% nonfat dry milk and incubated with primary antibodies against the following proteins: p53 (D07 DAKO), phospho-retinoblastoma (Sigma, St Louis, MO, USA), cyclins D1, E, A (Santa Cruz) and Beta-Actin (Sigma) as loading control. After washing in Tween-20 buffer, PVDF membranes were incubated with HRP secondary antibodies (Amersham, Piscataway, NJ, USA), and signal was detected using the ECL-plus reagent (Amersham) following the manufacture's instruction.

Results

Characterization of the MCF-7 $^{GFP\text{-}cetn2}$ cells to directly monitor centriole dynamics. We employed the MCF-7 $^{GFP\text{-}cetn2}$ breast cancer cells stably expressing GFP-centrin to directly monitor the timing of centriole duplication (37). MCF-7 cells represent an early breast cancer phenotype and are characterized by estrogen dependence for growth, wild-type p53, normal centrosome phenotype and low metastatic potential. Therefore, the MCF-7 cell line is an excellent model to study the relationship between mitogens, p53 status and regulation of the timing of centrosome duplication during cell cycle progression. The GFP-centrin2 chimera, expressed in the MCF-7 cells, is selectively incorporated into the structure of both centrioles making them clearly visible in living cells (Fig.1A). To assess whether the GFP-centrin2 construct affected the cell cycle profile of MCF-7 cells, we performed a FACS analysis of MCF-7 $^{GFP\text{-}cetn2}$ cells versus the parental cell line. Figure 1B shows that the two cell lines have nearly identical cell cycle profile with similar proportions of G_0/G_1, S and G_2/M phase cells. An advantage of using the MCF-7$^{GFP\text{-}cetn2}$ cells to monitor centrosome duplication is the ability to clear follow centriole dynamics during a complete cell cycle (Fig.1C-F). Based on GFP-centrin2 fluorescence and data presented in figure 2, cells in the G_0 phase of the cell cycle show a pair of centrioles closely adjacent to one another (Fig. 1C), while early G_1 cells are characterized by two centrioles that have separated a short distance from one another (Fig. 1D). G_1/S progression of the cell cycle is characterized by the formation of new centrioles originating adjacent to the pre-existing ones (Fig.1E), and finally cells in the G_2/M phase show two pair of centrioles, one pair at each mitotic spindle pole (Fig. 1F).

Centriole duplication is coupled with DNA replication in hormone stimulated MCF-7 $^{GFP\text{-}cetn2}$ cells through the orderly expression of G_1/S cyclins and progressive retinoblastoma phosphorylation. To determine the relationship between hormone stimulation, centriole duplication and cell cycle progression in breast cancer cells, MCF-7$^{GFP\text{-}cetn2}$ cells were synchronized in the G_o phase of the cell cycle by estrogen withdrawal. Following 48 hours starvation, cell cycle reentry was stimulated by addition of 17-β estradiol, EGF and IGF-I and the percentage of cells in each phase of the cell cycle was monitored by FACS analysis. MCF-7$^{GFP\text{-}cetn2}$ cells replicated their DNA following 24 hours hormone stimulation, progress from S to G2/M phase after 36 hours and became asynchronous by 48 hours (Fig. 2A). In order to determine the timing of centriole

duplication during cell cycle progression, we quantified the number of cells with 3 and 4 centrioles by counting GFP-centrin2 labeled spots (Fig. 2B). After 48 hours estrogen starvation, approximately 8% of MCF-7$^{GFP\text{-}cetn2}$ cells showed duplicated centrioles, indicating that centriole duplication is arrested in cells in G_o phase of the cell cycle. Following treatment with 17-β estradiol, EGF and IGF-I, the number of cells with duplicated centrioles increased progressively and by 24 hours ~56% of cells showed duplicated centrioles. These observations demonstrate that the process of centriole duplication is initiated before the onset of S phase but is coordinated with DNA replication during a complete cell cycle in the MCF-7 cells.

We then performed a time course immunoblotting analysis to determine if the coordination of centrosome duplication with DNA replication was linked to the orderly expression of G_1/S cyclins. The activity of G_1/S cyclins was determined by monitoring the phosphorylation status of the tumor suppressor retinoblastoma (Rb) (Fig. 3A). After 48 hours starvation, cyclin D1, E and A showed low levels of expression and Rb was in its active hypo-phosphorylated state. Following mitogen stimulation, cyclin D1 expression reached its maximum level by 8 hours followed by increase of cyclin E at 12 hours and cyclin A at 24 hours. The timing of G_1/S cyclin expression was linked to the progressive increase of Rb phosphorylation, which started after 4 hours of hormone stimulation and reached its maximum level by 24 hours. Taken together, these results demonstrate that in MCF-7 cells the timing of centriole duplication is coupled to DNA replication through the orderly expression of G1/S cyclins and consequent Rb inactivation.

Loss of the timing of centriole duplication is linked to deregulation of G_1/S cell cycle progression in MCF-7 cells with impaired p53 function. In order to determine if the coordination of centriole duplication and DNA replication during cell cycle progression was linked to the p53 status of MCF-7 cells, we employed a variant MCF-7 cell line with abrogated p53 (vMCF-7^{DNp53}). The cell cycle profile of cycling vMCF-7^{DNp53} cells showed ~55% of cells in the S phase of the cell cycle, indicating a higher proliferative activity compared to the parental cell line (~30%). After 48 hours estrogen starvation, the cell cycle profile of vMCF-7^{DNp53} cells was characterized by a decrease in S phase, albeit not as low as seen in the parental cell line. Nonetheless, this finding suggests that these cells still retained a partial hormone dependent phenotype regardless abrogation of p53 function. Interestingly, following hormone stimulation, the vMCF-7^{DNp53} cells showed a shortened G_1/S

cell cycle progression, characterized by earlier DNA replication, compared to the MCF-7$^{GFP\text{-}cetn2}$ cells (Fig. 2C). In order to determine if the acceleration of G_1/S phase progression was associated with deregulation of centrosome duplication, we performed an immunofluorescence analysis of vMCF-7^{DNp53} cells labeled with a monoclonal centrin2 antibody to determine the number of cells with duplicated centrioles. Compared to the parental cell line, cycling vMCF-7^{DNp53} cells showed a higher percentage of cells with duplicated centrosomes. After 48 hours estrogen starvation, ~ 40% of cells still retained duplicated centrioles and this phenotype was maintained during cell cycle reentry following hormone stimulation (Fig. 2D). Since in the MCF-7 cells centrosome duplication and DNA replication were coordinated through the orderly expression of cyclin D1, E and A, we investigated by immunoblotting the expression of G1/S cyclins and the phosphorylation status of Rb in the vMCF-7^{DNp53} cells (Fig. 3B). At all time points the vMCF-7^{Dnp53} cells showed higher level of mutant p53 compared to wild-type p53 expression in the parental cell line as expected (Fig. 3A and B). After 48 hours estrogen starvation, cyclin D1 levels were low, while cyclin E, A and phosphorylated Rb were expressed at significantly higher levels compared to the parental cells. Following mitogen stimulation of the vMCF-7^{DNp53} cells, while cyclin D1 maintained the expression timing in a similar fashion of MCF-7 cells, cyclin E and A were expressed at earlier time points leading to constitutive Rb phosphorylation. Taken together, these results indicate that deregulated expression of cyclin E and A is responsible of shortened G1/S cell cycle progression, robust Rb inactivation and loss of the timing of centriole duplication in the vMCF-7^{DNp53} cells.

17-β estradiol, EGF and IGF-I stimulation leads to centrosome amplification and abnormal mitotic spindles only in the vMCF-7^{DNp53} cells. To determine if abrogation of p53 function was also linked to the development of centrosome amplification following 17-Beta Estradiol, EGF and IGF-I stimulation in the MCF-7 cells, we quantified the number of cells with more than four centrioles in the MCF-7$^{GFP\text{-}cetn2}$ and vMCF-7^{DNp53} cells. Figure 4A shows that following starvation and hormone stimulated reentry in the cell cycle, centrosome amplification occurred only in the vMCF-7^{DNp53} cells. The highest percentage of cells with amplified centrosomes was observed 8 hours after mitogen stimulation and decreased by 24 hours due to centriole segregation during mitosis. Interestingly, the development of centrosome amplification was associated to the hyper-phosphorylated status of Rb due to deregulation of cyclin E and A expression (Fig. 3B). The

differences in the dynamics of centrosome duplication between MCF-7$^{GFP\text{-}cetn2}$ and vMCF-7^{DNp53} cells after starvation and 24 hours mitogen stimulation are illustrated in figure 4B-G. Cycling MCF-7$^{GFP\text{-}cetn2}$ showed most of cells with unduplicated centrioles (Fig. 4B). After 48 hours starvation, the centrioles were closed to one another, indicating an arrest in the G_o phase of the cell cycle (Fig. 4C). By 24 hours mitogen stimulation, the centrosome phenotype of MCF-7$^{GFP\text{-}cetn2}$ cells was characterized by duplicated centrioles (Fig. 4D). On the contrary, cycling vMCF-7^{DNp53} cells showed a duplicated centrosome phenotype (Fig. 4E). These cells also showed failure of arrest the centriole duplication cycle following 48 hours estrogen starvation (Fig. 4F). After 24 hours hormone stimulation, the vMCF-7^{DNp53} cells developed amplified centrosomes (Fig.4G).

Since the development of centrosome amplification is associated with chromosomal instability through the formation of aberrant mitoses, we quantified the number of bipolar, pseudobipolar and multipolar mitotic spindles in the MCF-7$^{GFP\text{-}cetn2}$ and vMCF-7^{DNp53} cells after 24 hours mitogen stimulation (Fig. 5A-D). Mitotic spindle morphology was characterized by immunofluorescence using antibodies directed against centrin-2 protein to label centrioles (green) and Aurora-A centrosome kinase to label mitotic spindles (red). Examples of mitotic figures in the vMCF-7^{DNp53} cells are illustrated in figure 5A-C. Bipolar spindles are characterized by the presence of two centrioles at each pole (Fig. 5A-A'). Pseudobipolar spindles show two poles where one of them or both contain more than two centrioles (Fig. 5B-B'), while the presence of more than two poles each containing two or more centrioles typify the multipolar mitotic spindles (Fig. 5C-C'). Interestingly, after 24 hours hormone stimulation, only the vMCF-7^{DNp53} cells showed a higher percentage of pseudobipolar and multipolar mitotic spindles compared to MCF-7$^{GFP\text{-}cetn2}$ cells (Fig. 5D). In conclusion, these results demonstrate that abrogation of p53 function deregulates the expression timing of cyclin E and A with perturbation of centrosome homeostasis in hormone stimulated MCF-7 cells. The development of centrosome amplification, through the generation of aberrant mitotic spindles, may represent the driven force in selecting cancer cell clones with more aggressive behavior.

Discussion

In this study, we investigated the relationship between mitogen and p53 signaling pathways in the coordination of centrosome duplication with cell cycle events in MCF-7 breast cancer cells. Several studies have demonstrated the role of 17-β Estradiol, EGF and IGF-I growth factors in the regulation of G_1/S phase progression in breast cancer derived cells (38-43). Following mitogen stimulation of G_o arrested cells, the reentry in the G_1 phase is governed by cyclin D1/cdk4 or cdk6 complex through the phosphorylation and consequent inactivation of the Rb protein (44, 45). Whereas cyclin D1 is essential for G_o/G_1 progression, cyclin E/cdk2 and cyclin A/cdk2 complexes are responsible for the G_1/S transition and onset of DNA replication during S phase, respectively (46-48).

Aberrant expression of G_1/S cyclins is linked to breast cancer development and progression (49-51). While cyclin D1 over expression is generally associated with an ERα+ phenotype and a favorable outcome, deregulation of cyclin E and A is linked to aneuploid, ERα- and metastatic breast carcinomas (52-57). Furthermore, it has been demonstrated that over expression of cyclin E and A induce centrosome amplification, suggesting an important role of these cyclins in the development of genomic instability (35, 58, 59). Interestingly, the effect of cyclin/cdk2 hyperactivity in the development of centrosome amplification has also been demonstrated by using small molecules cdk2 inhibitors able to rescue a normal centrosome phenotype in cancer cells (35, 60, 61). On the contrary, the tumor suppressor p53 preserves the stability of the genome by inhibiting centrosome reduplication. The ability of p53 in preventing centrosome reduplication in the absence of DNA replication is linked to over-expression of the cdk inhibitor p21, inhibition of cyclin/cdk2 activity and consequent activation of the G_1/S cell cycle checkpoint (35). However, how estrogen, growth factor and p53 pathways are mechanistically coupled with the activity of G1/S cyclins and the timing of centrosome duplication in breast cancer cells has not been established.

To address this issue, we employed MCF-7 cells over expressing a GFP-centrin2 chimera (MCF-$7^{GFP\text{-}cetn2}$) to directly monitor centriole duplication during cell cycle progression. In these cells, centriole duplication begins in late G_1 phase of the cell cycle and is coordinated with DNA replication following hormone stimulation. At a molecular level, the timing of centriole duplication was correlated with the orderly expression

of G1/S cyclins and progressive retinoblastoma phosphorylation. On the contrary, MCF-7 cells over expressing a mutated p53 protein (vMCF-7^{DNp53}) showed a shortened G_1/S phase progression following hormone stimulation. Although vMCF-7^{DNp53} cells still retained a hormone dependent phenotype, estrogen withdrawal was linked to loss of centriole duplication arrest. Following hormone stimulation, abrogation of p53 function did not affect the expression timing of cyclin D1, rather led to premature expression of cyclin E and A and consequent Rb hyper-phosphorylation. The phenotype of vMCF-7^{DNp53} cells was also characterized by the development of centrosome amplification leading to the generation of aberrant mitoses. The presence of pseudo- and multipolar mitotic spindles, responsible of chromosome missegregation, may represent the driven force for the development of chromosomal instability and phenotypic heterogeneity commonly observed in advanced breast carcinomas.

In accordance with these findings, it has been demonstrate that p53-null mammary cells generate a significant percentage of ERα- cancer cells arising from ERα+ breast carcinomas, suggesting that abrogation of p53 function accelerates the development of clonal heterogeneity in breast cancer (62). It has also been shown that estrogens promote the development of centrosome amplification and chromosomal instability in ACI rats, although the p53 status of these tumors has not been established (63). Our results demonstrate that ERα+ breast cancer cells with mutant p53 still require estrogens and growth factors to proliferate but are more susceptible to develop amplified centrosomes. Importantly, our study suggests a rationale for combining conventional anti-estrogen agents with small molecule inhibitors of cdk2 activity as a molecular targeted therapeutic approach to inhibit centrosome amplification and reduce consequent clonal heterogeneity in ERα+ breast carcinomas with mutant p53.

Acknowledgments

This work was supported by NCI CA72836 to JLS, USAMRMC BC022276 to ABD, the Mayo Clinic, and the Ph.D program in molecular oncology, University of Catania, Italy.

References

1. Ingle, J. N. Sequencing of endocrine therapy in postmenopausal women with advanced breast cancer. Clin Cancer Res, *10:* 362S-367S, 2004.
2. Nicholson, R. I. and Johnston, S. R. Endocrine therapy—current benefits and limitations. Breast Cancer Res Treat, *93 Suppl 1:* S3-10, 2005.
3. Lamote, I., Meyer, E., Massart-Leen, A. M., and Burvenich, C. Sex steroids and growth factors in the regulation of mammary gland proliferation, differentiation, and involution. Steroids, *69:* 145-159, 2004.
4. Anderson, E. and Clarke, R. B. Steroid receptors and cell cycle in normal mammary epithelium. J Mammary Gland Biol Neoplasia, *9:* 3-13, 2004.
5. Pietras, R. J., Levin, E. R., and Szego, C. M. Estrogen receptors and cell signaling. Science, *310:* 51-53, 2005.
6. Helguero, L. A., Faulds, M. H., Gustafsson, J. A., and Haldosen, L. A. Estrogen receptors alfa (ERalpha) and beta (ERbeta) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11. Oncogene, *24:* 6605-6616, 2005.
7. Cicatiello, L., Scafoglio, C., Altucci, L., Cancemi, M., Natoli, G., Facchiano, A., Iazzetti, G., Calogero, R., Biglia, N., De Bortoli, M., Sfiligoi, C., Sismondi, P., Bresciani, F., and Weisz, A. A genomic view of estrogen actions in human breast cancer cells by expression profiling of the hormone-responsive transcriptome. J Mol Endocrinol, *32:* 719-775, 2004.
8. Lee, Y. R., Park, J., Yu, H. N., Kim, J. S., Youn, H. J., and Jung, S. H. Up-regulation of PI3K/Akt signaling by 17beta-estradiol through activation of estrogen receptor-alpha, but not estrogen receptor-beta, and stimulates cell growth in breast cancer cells. Biochem Biophys Res Commun, *336:* 1221-1226, 2005.
9. Calaf, G. M. Susceptibility of human breast epithelial cells in vitro to hormones and drugs. Int J Oncol, *28:* 285-295, 2006.
10. Strange, K. S., Wilkinson, D., and Emerman, J. T. Mitogenic properties of insulin-like growth factors I and II, insulin-like growth factor binding protein-3 and epidermal growth factor on human breast epithelial cells in primary culture. Breast Cancer Res Treat, *75:* 203-212, 2002.
11. Russo, J., Hu, Y. F., Yang, X., and Russo, I. H. Developmental, cellular, and molecular basis of human breast cancer. J Natl Cancer Inst Monogr*:* 17-37, 2000.
12. Geffroy, N., Guedin, A., Dacquet, C., and Lefebvre, P. Cell cycle regulation of breast cancer cells through estrogen-induced activities of ERK and Akt protein kinases. Mol Cell Endocrinol, *237:* 11-23, 2005.
13. Stoica, G. E., Franke, T. F., Moroni, M., Mueller, S., Morgan, E., Iann, M. C., Winder, A. D., Reiter, R., Wellstein, A., Martin, M. B., and Stoica, A. Effect of estradiol on estrogen receptor-alpha gene

expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. Oncogene, *22:* 7998-8011, 2003.

14. Lengauer, C., Kinzler, K. W., and Vogelstein, B. Genetic instabilities in human cancers. Nature, *396:* 643-649., 1998.
15. Lingle, W. L., Barrett, S. L., Negron, V. C., D'Assoro, A. B., Boeneman, K., Liu, W., Whitehead, C. M., Reynolds, C., and Salisbury, J. L. Centrosome amplification drives chromosomal instability in breast tumor development. Proc Natl Acad Sci U S A, *99:* 1978-1983, 2002.
16. Fukasawa, K. Centrosome amplification, chromosome instability and cancer development. Cancer Lett, *230:* 6-19, 2005.
17. Suizu, F., Ryo, A., Wulf, G., Lim, J., and Lu, K. P. Pin1 regulates centrosome duplication, and its overexpression induces centrosome amplification, chromosome instability, and oncogenesis. Mol Cell Biol, *26:* 1463-1479, 2006.
18. Brinkley, B. R. and Goepfert, T. M. Supernumerary centrosomes and cancer: Boveri's hypothesis resurrected. Cell Motil Cytoskeleton, *41:* 281-288, 1998.
19. Salisbury, J. L., Suino, K. M., Busby, R., and Springett, M. Centrin-2 is required for centriole duplication in mammalian cells. Curr Biol, *12:* 1287-1292, 2002.
20. Sluder, G. and Hinchcliffe, E. H. The coordination of centrosome reproduction with nuclear events during the cell cycle. *In:* Centrosome in Cell Replication and Early Development, Vol. 49, pp. 267-289. 2000.
21. Meraldi, P., Lukas, J., Fry, A. M., Bartek, J., and Nigg, E. A. Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. Nat Cell Biol, *1:* 88-93, 1999.
22. Balczon, R., Bao, L., Zimmer, W. E., Brown, K., Zinkowski, R. P., and Brinkley, B. R. Dissociation of centrosome replication events from cycles of DNA synthesis and mitotic division in hydroxyurea-arrested Chinese hamster ovary cells. J Cell Biol, *130:* 105-115, 1995.
23. D'Assoro, A. B., Barrett, S. L., Folk, C., Negron, V. C., Boeneman, K., Busby, R. C., Whitehead, C. M., Stivala, F., Lingle, L. L., and Salisbury, J. L. Amplified centrosomes in breast cancer: a potential indicator of tumor aggressiveness. Breast Cancer Res Treat, *75:* 25-34, 2002.
24. Lingle, W. L., Lutz, W. H., Ingle, J. N., Maihle, N. J., and Salisbury, J. L. Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. Proc Natl Acad Sci U S A, *95:* 2950-2955, 1998.
25. Zhang, H., Shi, X., Paddon, H., Hampong, M., Dai, W., and Pelech, S. B23/nucleophosmin serine 4 phosphorylation mediates mitotic functions of polo-like kinase 1. J Biol Chem, *279:* 35726-35734, 2004.

26. Daniels, M. J., Wang, Y., Lee, M., and Venkitaraman, A. R. Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. Science, *306:* 876-879, 2004.
27. D'Assoro, A. B., Lingle, W. L., and Salisbury, J. L. Centrosome amplification and the development of cancer. Oncogene, *21:* 6146-6153, 2002.
28. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G. F. Abnormal centrosome amplification in the absence of p53. Science, *271:* 1744-1747, 1996.
29. Hernando, E., Nahle, Z., Juan, G., Diaz-Rodriguez, E., Alaminos, M., Hemann, M., Michel, L., Mittal, V., Gerald, W., Benezra, R., Lowe, S. W., and Cordon-Cardo, C. Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature, *430:* 797-802, 2004.
30. Meraldi, P., Lukas, J., Fry, A., Bartek, J., and Nigg, E. Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. Nature Cell Biol, *1:* 88-93, 1999.
31. Mussman, J. G., Horn, H. F., Carroll, P. E., Okuda, M., Tarapore, P., Donehower, L. A., and Fukasawa, K. Synergistic induction of centrosome hyperamplification by loss of p53 and cyclin E overexpression. Oncogene, *19:* 1635-1646, 2000.
32. Hinchcliffe, E. H. and Sluder, G. Two for two: Cdk2 and its role in centrosome doubling. Oncogene, *21:* 6154-6160, 2002.
33. Liu, X. and Erikson, R. L. Activation of Cdc2/cyclin B and inhibition of centrosome amplification in cells depleted of Plk1 by siRNA. PNAS, *99:* 8672-8676, 2002.
34. Giet, R., Petretti, C., and Prigent, C. Aurora kinases, aneuploidy and cancer, a coincidence or a real link? Trends Cell Biol, *15:* 241-250, 2005.
35. D'Assoro, A. B., Busby, R., Suino, K., Delva, E., Almodovar-Mercado, G. J., Johnson, H., Folk, C., Farrugia, D. J., Vasile, V., Stivala, F., and Salisbury, J. L. Genotoxic stress leads to centrosome amplification in breast cancer cell lines that have an inactive G1/S cell cycle checkpoint. Oncogene, *23:* 4068-4075, 2004.
36. Bennett, R. A., Izumi, H., and Fukasawa, K. Induction of centrosome amplification and chromosome instability in p53-null cells by transient exposure to subtoxic levels of S-phase-targeting anticancer drugs. Oncogene, *23:* 6823-6829, 2004.
37. D'Assoro, A. B., Stivala, F., Barrett, S., Ferrigno, G., and Salisbury, J. L. GFP-centrin as a marker for centriole dynamics in the human breast cancer cell line MCF-7. Ital J Anat Embryol, *106(2:* 103-110., 2001.
38. Felty, Q., Singh, K. P., and Roy, D. Estrogen-induced G1/S transition of G0-arrested estrogen-dependent breast cancer cells is regulated by mitochondrial oxidant signaling. Oncogene, *24:* 4883-4893, 2005.
39. Planas-Silva, M. D. and Weinberg, R. A. Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. Mol Cell Biol, *17:* 4059-4069, 1997.

40. Nass, S. J. and Dickson, R. B. Epidermal growth factor-dependent cell cycle progression is altered in mammary epithelial cells that overexpress c-myc. Clin Cancer Res, *4:* 1813-1822, 1998.
41. Guo, J., Sheng, G., and Warner, B. W. Epidermal growth factor-induced rapid retinoblastoma phosphorylation at Ser780 and Ser795 is mediated by ERK1/2 in small intestine epithelial cells. J Biol Chem, *280:* 35992-35998, 2005.
42. Hamelers, I. H., Van Schaik, R. F., Sussenbach, J. S., and Steenbergh, P. H. 17beta-Estradiol responsiveness of MCF-7 laboratory strains is dependent on an autocrine signal activating the IGF type I receptor. Cancer Cell Int, *3:* 10, 2003.
43. Furstenberger, G. and Senn, H. J. Insulin-like growth factors and cancer. Lancet Oncol, *3:* 298-302, 2002.
44. Grillo, M., Bott, M. J., Khandke, N., McGinnis, J. P., Miranda, M., Meyyappan, M., Rosfjord, E. C., and Rabindran, S. K. Validation of cyclin D1/CDK4 as an anticancer drug target in MCF-7 breast cancer cells: Effect of regulated overexpression of cyclin D1 and siRNA-mediated inhibition of endogenous cyclin D1 and CDK4 expression. Breast Cancer Res Treat*:* 1-10, 2005.
45. Qiu, C., Shan, L., Yu, M., and Snyderwine, E. G. Deregulation of the cyclin D1/Cdk4 retinoblastoma pathway in rat mammary gland carcinomas induced by the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Cancer Res, *63:* 5674-5678, 2003.
46. Prall, O. W., Sarcevic, B., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J Biol Chem, *272:* 10882-10894, 1997.
47. Mailand, N. and Diffley, J. F. CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. Cell, *122:* 915-926, 2005.
48. Hwang, H. C. and Clurman, B. E. Cyclin E in normal and neoplastic cell cycles. Oncogene, *24:* 2776-2786, 2005.
49. Akli, S., Zheng, P. J., Multani, A. S., Wingate, H. F., Pathak, S., Zhang, N., Tucker, S. L., Chang, S., and Keyomarsi, K. Tumor-specific low molecular weight forms of cyclin E induce genomic instability and resistance to p21, p27, and antiestrogens in breast cancer. Cancer Res, *64:* 3198-3208, 2004.
50. Bindels, E. M., Lallemand, F., Balkenende, A., Verwoerd, D., and Michalides, R. Involvement of G1/S cyclins in estrogen-independent proliferation of estrogen receptor-positive breast cancer cells. Oncogene, *21:* 8158-8165, 2002.
51. Musgrove, E. A. Cyclins: roles in mitogenic signaling and oncogenic transformation. Growth Factors, *24:* 13-19, 2006.

52. Joe, A. K., Memeo, L., McKoy, J., Mansukhani, M., Liu, H., Avila-Bront, A., Romero, J., Li, H., Troxel, A., and Hibshoosh, H. Cyclin D1 overexpression is associated with estrogen receptor expression in Caucasian but not African-American breast cancer. Anticancer Res, *25:* 273-281, 2005.
53. Lebeau, A., Unholzer, A., Amann, G., Kronawitter, M., Bauerfeind, I., Sendelhofert, A., Iff, A., and Lohrs, U. EGFR, HER-2/neu, cyclin D1, p21 and p53 in correlation to cell proliferation and steroid hormone receptor status in ductal carcinoma in situ of the breast. Breast Cancer Res Treat, *79:* 187-198, 2003.
54. Park, K., Han, S., Kim, H. Y., and Ko, I. Cytologic evaluation of cyclin D1 expression in primary breast carcinoma. Cancer, *93:* 211-215, 2001.
55. Span, P. N., Tjan-Heijnen, V. C., Manders, P., Beex, L. V., and Sweep, C. G. Cyclin-E is a strong predictor of endocrine therapy failure in human breast cancer. Oncogene, *22:* 4898-4904, 2003.
56. Nielsen, A. L. and Nyholm, H. C. The combination of p53 and age predict cancer specific death in advanced stage (FIGO Ic-IV) of endometrial carcinoma of endometrioid type. An immunohistochemical examination of growth fraction: Ki-67, MIB-1 and PC10; suppressor oncogene protein: p53; oncogene protein: p185 and age, hormone treatment, stage, and histologic grade. Eur J Obstet Gynecol Reprod Biol, *70:* 79-85, 1996.
57. Michalides, R., van Tinteren, H., Balkenende, A., Vermorken, J. B., Benraadt, J., Huldij, J., and van Diest, P. Cyclin A is a prognostic indicator in early stage breast cancer with and without tamoxifen treatment. Br J Cancer, *86:* 402-408, 2002.
58. Kronenwett, U., Huwendiek, S., Ostring, C., Portwood, N., Roblick, U. J., Pawitan, Y., Alaiya, A., Sennerstam, R., Zetterberg, A., and Auer, G. Improved grading of breast adenocarcinomas based on genomic instability. Cancer Res, *64:* 904-909, 2004.
59. Kronenwett, U., Castro, J., Roblick, U. J., Fujioka, K., Ostring, C., Faridmoghaddam, F., Laytragoon-Lewin, N., Tribukait, B., and Auer, G. Expression of Cyclins A, E and Topoisomerase II alpha correlates with centrosome amplification and genomic instability and influences the reliability of cytometric S-phase determination. BMC Cell Biol, *4:* 8, 2003.
60. Duensing, S., Duensing, A., Lee, D. C., Edwards, K. M., Piboonniyom, S. O., Manuel, E., Skaltsounis, L., Meijer, L., and Munger, K. Cyclin-dependent kinase inhibitor indirubin-3'-oxime selectively inhibits human papillomavirus type 16 E7-induced numerical centrosome anomalies. Oncogene, *23:* 8206-8215, 2004.
61. Duensing, A., Liu, Y., Tseng, M., Malumbres, M., Barbacid, M., and Duensing, S. Cyclin-dependent kinase 2 is dispensable for normal centrosome duplication but required for oncogene-induced centrosome overduplication. Oncogene, 2006.

62. Medina, D., Kittrell, F. S., Shepard, A., Contreras, A., Rosen, J. M., and Lydon, J. Hormone dependence in premalignant mammary progression. Cancer Res, *63:* 1067-1072, 2003.

63. Li, J. J., Weroha, S. J., Lingle, W. L., Papa, D., Salisbury, J. L., and Li, S. A. Estrogen mediates Aurora-A overexpression, centrosome amplification, chromosomal instability, and breast cancer in female ACI rats. Proc Natl Acad Sci U S A, *101:* 18123-18128, 2004.

Figure Legends

Figure 1. Fluorescence microscopy of GFP-centrin2 localization and cell cycle profile in MCF-7 cells. (A) A field of cycling MCF-7$^{GFP\text{-}cetn2}$ cells showing different centriole duplication stages with one pair of centrin-labeled or two pair of duplicated centrioles. (B) Cell cycle analysis based on flow-cytometry (FACS) for MCF-7 and MCF-7$^{GFP\text{-}cetn2}$ cells. Both cell populations show a similar proportion of cells in each cell cycle stage. GFP-centrin2 labeled centrioles (C-F). (C) G_o phase with a pair of centrioles adjacent to one another. (D) Early G1 phase with centrioles that have separated from one another. (E) Late G1 or early S phase characterized by the beginning of centriole duplication. (F) G2/M phase with duplicated pairs of centrioles at each mitotic spindle pole.

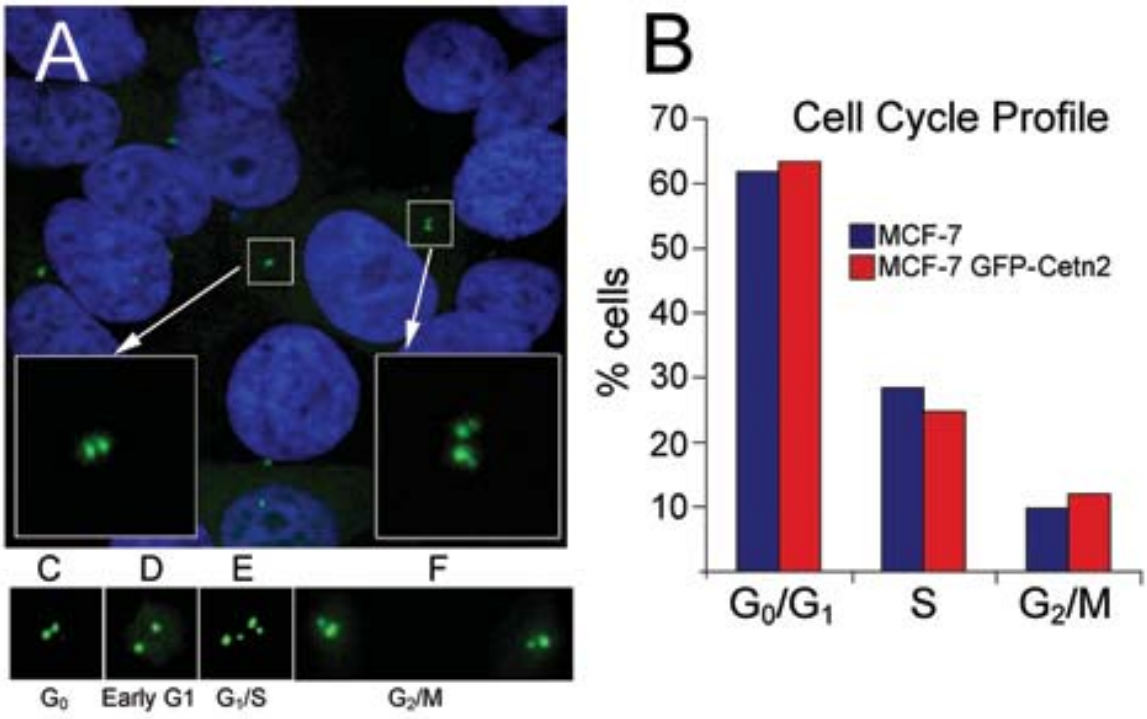


Figure 2. Cell cycle profile and centriole duplication timing in the MCF-7$^{GFP\text{-}cetn2}$ and vMCF-7^{DNP53}cells. (A) FACS analysis of cell cycle progression following starvation and hormone stimulation in the MCF-7$^{GFP\text{-}cetn2}$ cells. Cycling cells were synchronized in the G_o phase of the cell cycle by 48 hours estrogen withdrawal. Cell cycle reentry was stimulated by addition of 17-β Estradiol, EGF and IGF-I, MCF-7$^{GFP\text{-}cetn2}$ cells replicate their DNA after 24 hours hormone stimulation, progress from S to G2/M phase after 36 hours and became asynchronous by 48 hours. (B) Induction of centriole duplication after hormone stimulation in the MCF-7$^{GFP\text{-}cetn2}$ cells. MCF-7 cells with 3 and 4 GFP-centrin2 labeled spots were counted as cells with duplicated centrioles. Following 48 hours estrogen starvation, 8% of cells showed duplicated centrioles, while the number of MCF-7$^{GFP\text{-}cetn2}$ cells with duplicated centrioles increased progressively after mitogen stimulation. (C) FACS analysis of cell cycle progression following time course hormone stimulation in the vMCF-7^{DNP53}cells. Cycling vMCF-7^{DNP53}cells showed higher percentage of cells in the S phase compared to the parental cell line. After 48 hours estrogen starvation, there was a reduction of cells in the S phase of the cell cycle. Following mitogen stimulation, vMCF-7^{DNP53}cells showed a shortened G1/S cell cycle progression, characterized by earlier DNA replication compared to the parental cells. (D) Centriole duplication profile after hormone stimulation in the vMCF-7^{DNP53}cells. Duplicated centrioles were determined by immunofluorescence analysis of vMCF-7^{DNP53}cells labeled with a monoclonal antibody directed against the protein centrin2. Compared to the parental cell line, cycling vMCF-7^{DNP53} cells showed a higher percentage of cells with duplicated centrosomes at all time points in the immunofluorescence analysis. After 48 hours estrogen withdrawal, ~40% of cells still retained duplicated centrioles and this phenotype was maintained during the progression through the cell cycle following mitogen stimulation.

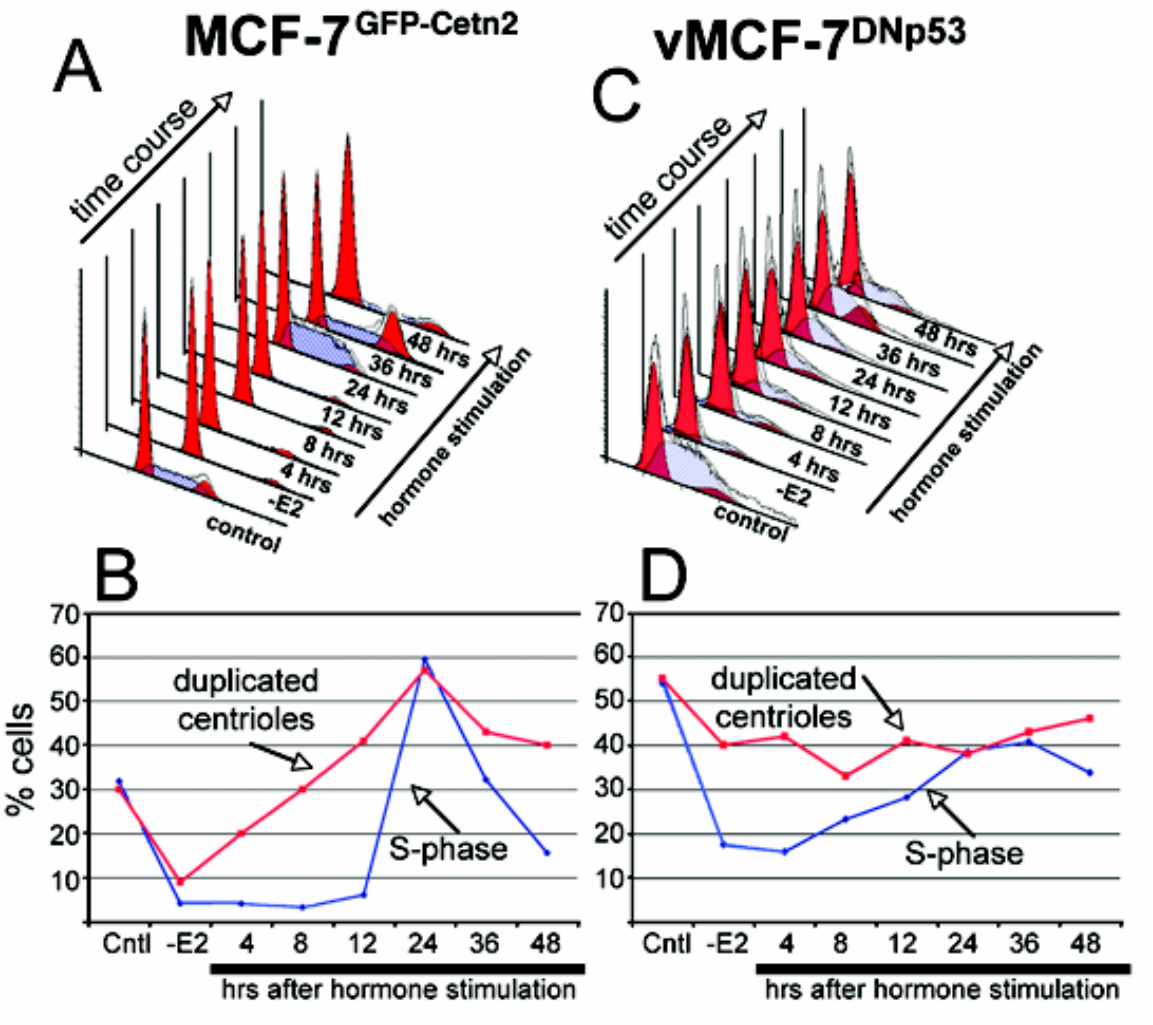

A
MCF-7 GFP-Cetn2
time course
48 hrs
36 hrs
24 hrs
12 hrs
8 hrs
4 hrs
-E2
control
hormone stimulation
C
vMCF-7 DNp53
time course
48 hrs
36 hrs
24 hrs
12 hrs
8 hrs
4 hrs
-E2
control
hormone stimulation
B
% cells
duplicated centrioles
S-phase
Cntl -E2 4 8 12 24 36 48
hrs after hormone stimulation
D
duplicated centrioles
S-phase
Cntl -E2 4 8 12 24 36 48
hrs after hormone stimulation

Figure 3. (A-B) Immunoblotting analysis of MCF-7$^{GFP\text{-}cetn2}$ and vMCF-7^{DNP53}cells. The abundance of p53, G1/S cyclins and phospho-Rb was analyzed following 48 hours estrogen withdrawal (-E2) and time course (4, 8, 12, 24, 36 and 48 hours) 17-β estradiol, EGF and IGF-I stimulation. Whole-cell lysate (20μg protein) was loaded in each lane. β-Actin was used as loading control.

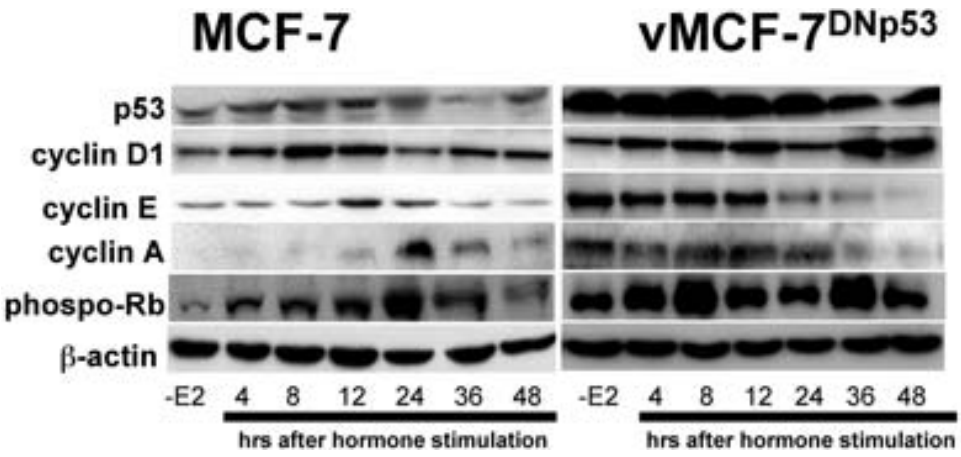


Figure 4. Analysis of centrosome amplification in the MCF-7$^{GFP\text{-}cetn2}$ and vMCF-7^{DNP53}cells. (A) Graph showing the percentage of cells with more than four centrioles after 48 hours estrogen starvation and time course hormone stimulation. Centrosome amplification occurred only in the vMCF-7^{DNP53}cells showing the highest percentage of amplified centrosomes 8 hours following mitogen stimulation. (B-D) Centrosome phenotype in cycling MCF-7$^{GFP\text{-}cetn2}$ cells, after estrogen starvation and 24 hours hormone stimulation. Centrioles were labeled in green by the GFP-centrin2 construct, pericentriolar material (PCM) was labeled in red using a polyclonal antibody against the protein pericentrin, while nuclei were stained in blue with Hoescht dye at 1µg/ml. (E-G) Immunofluorescence of centrosomes in cycling vMCF-7^{DNP53}cells, after estrogen starvation and 24 hours hormone stimulation. Centrioles and pericentriolar material (PCM) were labeled for centrin2 (green) and pericentrin (red), respectively. Nuclei were stained in blue with Hoescht dye at 1µg/ml.

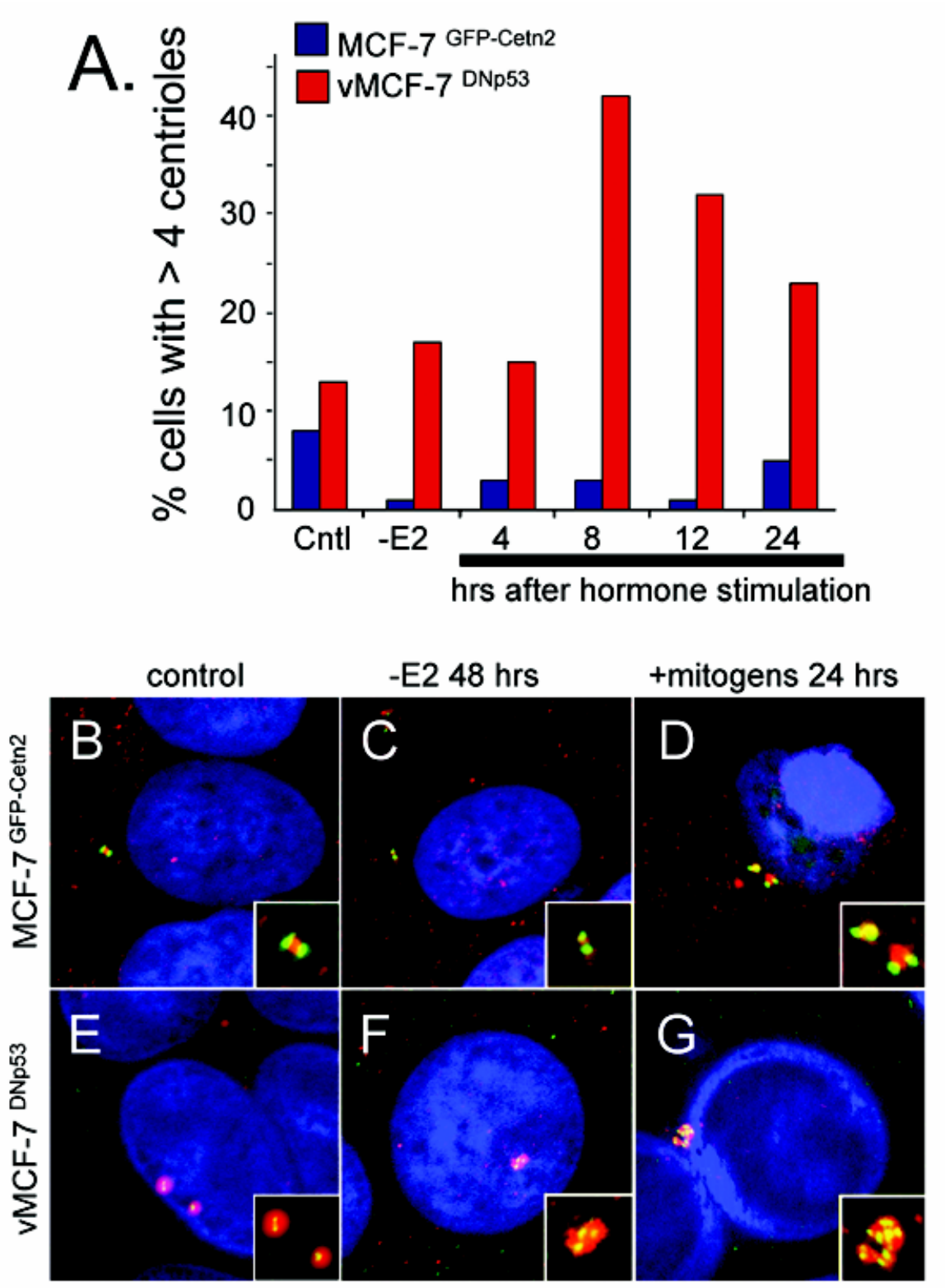
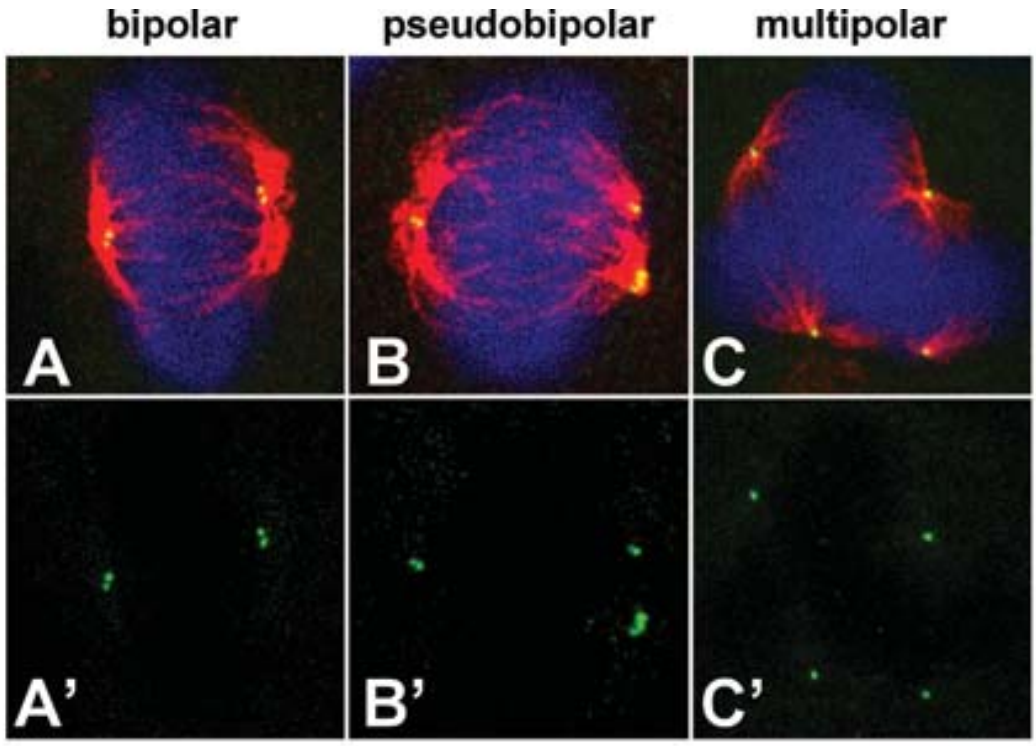
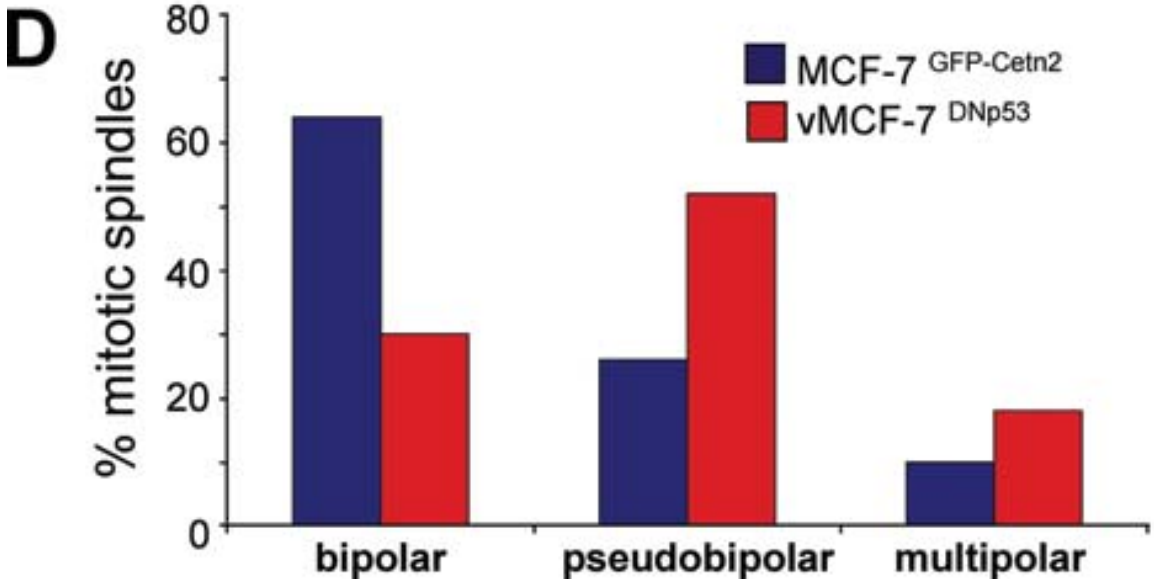

A.
MCF-7 GFP-Cetn2
vMCF-7 DNp53
% cells with > 4 centrioles
0
10
20
30
40
Cntl
-E2
4
8
12
24
hrs after hormone stimulation
control
-E2 48 hrs
+mitogens 24 hrs
MCF-7 GFP-Cetn2
vMCF-7 DNp53
B
C
D
E
F
G

Figure 5. Characterization of mitotic spindle morphology in MCF-7 $^{GFP\text{-}cetn2}$ and vMCF-7^{DNP53} cells. (A-C) Immunofluorescence of bipolar, pseudobipolar and multipolar mitotic spindles in the vMCF-7^{DNP53}cells. Centrioles were labeled in green with a monoclonal antibody for centrin2 (A'-C'), mitotic spindles were labeled in red with a polyclonal antibody for the centrosome kinase aurora-A. DNA content was labeled in blue with Hoescht dye at 1µg/ml.DAPI. (D) Graph showing the percentage of mitotic spindles with bipolar, pseudobipolar and multipolar morphology in MCF-7$^{GFP\text{-}cetn2}$ and vMCF-7^{DNP53} cells following 24 hours 17-β Estradiol, EGF and IGF-I stimulation. vMCF-7^{DNP53} cells showed a higher percentage of abnormal mitotic spindles compared to the MCF-7$^{GFP\text{-}cetn2}$ cells.





CONCLUSIONS

The results reported in this grant suggest that the development and progression of breast cancer is a complex process involving the role of estrogens, growth factor signaling pathways and abrogation of the p53 protein leading to inactivation of the G1/S cell cycle checkpoint. In particular, the abrogation of the p53 pathway plays a key role in the development of centrosome amplification following mitogens stimulation or induction of genotoxic stress through deregulation of cyclin/cdk2 complex and over-expression of the centrosome kinase Aurora-A. These findings are important to understand if the process of tumorigenesis that characterize breast cancer development and progression is marked by loss of cell cycle checkpoints and consequent induction of centrosome amplification. In turn, the development of centrosome amplification and chromosomal instability is essential in conferring to the tumor cells high metastatic potential and chemoresistant properties with cathastrophic consequences for breast cancer patients. We also suggest that centrosome amplification may represent a suitable marker to monitor tumor aggressiveness and a novel molecular therapeutic target for the treatment of breast carcinomas resistant to conventional anticancer drugs.

References:

1. Lengauer, C., K.W. Kinzler, and B. Vogelstein, Genetic instabilities in human cancers. Nature, 1998. 396(6712): p. 643-9.

2. Lengauer, C., K. Kinzler, and B. Vogelstein, Genetic instability in colorectal cancers. Nature, 1997. 386: p. 623-627.
3. Kronenwett, U., S. Huwendiek, C. Ostring, N. Portwood, U.J. Roblick, Y. Pawitan, A. Alaiya, R. Sennerstam, A. Zetterberg, and G. Auer, Improved Grading of Breast Adenocarcinomas Based on Genomic Instability. Cancer Res, 2004. 64(3): p. 904-909.
4. Lingle, W.L., S.L. Barrett, V.C. Negron, A.B. D'Assoro, K. Boeneman, W. Liu, C.M. Whitehead, C. Reynolds, and J.L. Salisbury, Centrosome amplification drives chromosomal instability in breast tumor development. Proc Natl Acad Sci U S A, 2002. 99: p. 1978-1983.
5. Dey, P., Aneuploidy and malignancy: an unsolved equation. J Clin Pathol, 2004. 57(12): p. 1245-9.
6. Russo, J., Y.-F. Hu, Yang, X., and I.H. Russo, Developmental, cellular, and molecular basis of human breast cancer. J. Natl. Cancer Instit. Monographs, 2000. 27: p. 17-37.
7. Li, Q., S. Ahmed, and J.A. Loeb, Development of an autocrine neuregulin signaling loop with malignant transformation of human breast epithelial cells. Cancer Res, 2004. 64(19): p. 7078-85.
8. Thor, A. and D. Yandell, Molecular Pathology of Breast Carcinoma, in Diseases of the Breast, J. Harris, et al., Editors. 1996, Lippincott-Raven: Philadelphia. p. 445-454.
9. Khan, S.A., M.A. Rogers, K.K. Khurana, M.M. Meguid, and P.J. Numann, Estrogen receptor expression in benign breast epithelium and breast cancer risk. J Natl Cancer Inst, 1998. 90(1): p. 37-42.
10. Gradishar, W.J., S.B. Wedam, M. Jahanzeb, J. Erban, S.A. Limentani, K.T. Tsai, S.R. Olsen, and S.M. Swain, Neoadjuvant docetaxel followed by adjuvant doxorubicin and cyclophosphamide in patients with stage III breast cancer. Ann Oncol, 2005.
11. Fetting, J.H., R. Gray, D.L. Fairclough, T.J. Smith, K.A. Margolin, M.L. Citron, M. Grove-Conrad, D. Cella, K. Pandya, N. Robert, I.C. Henderson, C.K. Osborne, and M.D. Abeloff, Sixteen-week multidrug regimen versus cyclophosphamide, doxorubicin, and fluorouracil as adjuvant therapy for node-positive, receptor-negative breast cancer: an Intergroup study. J Clin Oncol, 1998. 16(7): p. 2382-91.
12. Batist, G., Anthracyclines. Cancer Chemother Biol Response Modif, 2001. 19: p. 47-58.
13. Colleoni, M., S. Li, R.D. Gelber, A.S. Coates, M. Castiglione-Gertsch, K.N. Price, J. Lindtner, C.M. Rudenstam, D. Crivellari, J. Collins, O. Pagani, E. Simoncini, B. Thurlimann, E. Murray, J. Forbes, D. Erzen, S. Holmberg, A. Veronesi, and A. Goldhirsch, Timing of CMF chemotherapy in combination with tamoxifen in postmenopausal women with breast cancer: role of endocrine responsiveness of the tumor. Ann Oncol, 2005. 16(5): p. 716-25.
14. Geisler, S., P.E. Lonning, T. Aas, H. Johnsen, O. Fluge, D.F. Haugen, J.R. Lillehaug, L.A. Akslen, and A.L. Borresen-Dale, Influence of TP53 gene alterations and c-erbB-2 expression on the response to treatment with doxorubicin in locally advanced breast cancer. Cancer Res, 2001. 61(6): p. 2505-12.
15. Kaufmann, S.H. and D.L. Vaux, Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. Oncogene, 2003. 22(47): p. 7414-30.
16. Doyle, L.A. and D.D. Ross, Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene, 2003. 22(47): p. 7340-58.
17. Lenning, P.E., Study of suboptimum treatment response: lessons from breast cancer. Lancet Oncol, 2003. 4: p. 177-185.
18. Brinkley, B.R. and T.M. Goepfert, Supernumerary centrosomes and cancer: Boveri's hypothesis resurrected. Cell Motil Cytoskeleton, 1998. 41(4): p. 281-8.
19. Lingle, W.L., W.H. Lutz, J.N. Ingle, N.J. Maihle, and J.L. Salisbury, Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. Proc Natl Acad Sci U S A, 1998. 95(6): p. 2950-5.
20. Sluder, G. and E.H. Hinchcliffe, The coordination of centrosome reproduction with nuclear events during the cell cycle, in Centrosome in Cell Replication and Early Development. 2000. p. 267-289.
21. Balczon, R., L. Bao, W.E. Zimmer, K. Brown, R.P. Zinkowski, and B.R. Brinkley, Dissociation of centrosome replication events from cycles of DNA synthesis and mitotic division in hydroxyurea-arrested Chinese hamster ovary cells. J Cell Biol, 1995. 130(1): p. 105-15.
22. D'Assoro, A.B., R. Busby, K. Suino, E. Delva, G.J. Almodovar-Mercado, H. Johnson, C. Folk, D.J. Farrugia, V. Vasile, F. Stivala, and J.L. Salisbury, Genotoxic stress leads to centrosome amplification in breast cancer cell lines that have an inactive G1/S cell cycle checkpoint. Oncogene, 2004. 23(23): p. 4068-4075.

23. Meraldi, P., J. Lukas, A.M. Fry, J. Bartek, and E.A. Nigg, Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. Nat Cell Biol, 1999. 1(2): p. 88-93.
24. Harper, J.D., M.A. Sanders, and J.L. Salisbury, Phosphorylation of nuclear and flagellar basal apparatus proteins during flagellar regeneration in Chlamydomonas reinhardtii. J Cell Biol, 1993. 122(4): p. 877-86.
25. Hinchcliffe, E.H. and G. Sluder, Two for two: Cdk2 and its role in centrosome doubling. Oncogene, 2002. 21(40): p. 6154-60.
26. Fukasawa, K., T. Choi, R. Kuriyama, S. Rulong, and G.F. Vande Woude, Abnormal centrosome amplification in the absence of p53. Science, 1996. 271(5256): p. 1744-7.
27. Mussman, J.G., H.F. Horn, P.E. Carroll, M. Okuda, P. Tarapore, L.A. Donehower, and K. Fukasawa, Synergistic induction of centrosome hyperamplification by loss of p53 and cyclin E overexpression. Oncogene, 2000. 19(13): p. 1635-46.
28. Liu, X. and R.L. Erikson, Activation of Cdc2/cyclin B and inhibition of centrosome amplification in cells depleted of Plk1 by siRNA. PNAS, 2002. 99(13): p. 8672-8676.
29. Giet, R., C. Petretti, and C. Prigent, Aurora kinases, aneuploidy and cancer, a coincidence or a real link? Trends Cell Biol, 2005. 15(5): p. 241-50.
30. Lingle, W.L. and J.L. Salisbury, Altered centrosome structure is associated with abnormal mitoses in human breast tumors. Am J Pathol, 1999. 155(6): p. 1941-51.
31. Doxsey, S., The centrosome--a tiny organelle with big potential [news; comment]. Nat Genet, 1998. 20(2): p. 104-6.
32. Duensing, S. and K. Munger, Centrosomes, genomic instability, and cervical carcinogenesis. Crit Rev Eukaryot Gene Expr, 2003. 13(1): p. 9-23.
33. Ghadimi, B.M., D.L. Sackett, M.J. Difilippantonio, E. Schrock, T. Neumann, A. Jauho, G. Auer, and T. Ried, Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations. Genes Chromosomes Cancer, 2000. 27(2): p. 183-90.
34. D'Assoro, A.B., W.L. Lingle, and J.L. Salisbury, Centrosome amplification and the development of cancer. Oncogene, 2002. 21(40): p. 6146-53.
35. Bennett, R.A., H. Izumi, and K. Fukasawa, Induction of centrosome amplification and chromosome instability in p53-null cells by transient exposure to subtoxic levels of S-phase-targeting anticancer drugs. Oncogene, 2004. 23(40): p. 6823-9.